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Journal of Advanced Scientific Research

J.Adv.Sci.Res, 2010, 1(2); 12-18

Research Article

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ISSN: 0976-9595

Synthesis, Characterization, Anti-Tumor and Anti-Microbial Activity of Fatty Acid Analog of 2, 6-Diisopropylphenol

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ABSTRACT

Derivative of 2, 6-diisopropylphenol (propofol) was prepared by coupling with 9-hydroxy-11-Z-octadecenoic acid (isolated from seed oil of *Holarrhena antidysenterica)* with the C_1-α-hydroxy function of 2, 6-diisopropylphenol. The coupling reaction between fatty acid and propofol was carried out by dicyclohexylcarbodiimide in the presence of catalytic amount of dimethylaminopyridine to produce quantitative yield of the desired product. Spectroscopic studies confirmed the formation of the desired product. The compound was then investigated for its *in-vitro* anticancer activity against a panel of solid human tumor cell lines including HeLa, MCF-7 and HL-60(human leukemia) cells. Its cytotoxicity was also determined against non-cancerous mammalian cells (VERO cells) for comparison. The analog was cytotoxic against all cancer cell lines whereas no effect was observed against normal cells. The compound was also screened for its antimicrobial activity against *E. coli*, *S. aureus* and *S. albus*. The compound show good antimicrobial activity against *E. coli* and *S. albus*.

Keywords: Propofol, Holarrhena antidysenterica, Cytotoxicity, Anti-microbial activity, Anti-tumor activity

INTRODUCTION

Propofol (2, 6-diisopropylphenol) is a hypnotic alkyl phenol derivative. It is the most extensively used general anesthetic-sedative agent[1, 2]. At high levels, it is non-toxic to humans[3] (3 to 8µg/ml; 20 to 50µM), this agent is associated with minimal respiratory depression and has a short half life with duration of action of 2-10 minutes. It is a global central nervous system depressant. It decreases cerebral oxygen consumption, reduces intracranial pressure and has potent anti-convulsant properties[4]. It is a potent antioxidant[5-8] and has been shown to stimulate protein kinase C[9, 10], inhibit calcium entry in muscle cells[11] and increase the calcium sensitivity of myofilaments in ventricular myocytes[12]. It is a potent bronchodilator and has anti-inflammatory properties. As a consequence of these properties, propofol is being increasingly used in the management of traumatic head injury, status epilepticus, delirium tremens, and status asthmaticus and in critically ill septic patients[13]. 3 to 8µg/ml concentrations of propofol were reported to decrease the metastatic potential of human cancer cells, including HeLa, H71080, HOS and RPMI-7951 cells[14]. Propofol was reported to inhibit pulmonary metastasis of murine osteosarcoma (CM8) cells in mice through the modulation of Rho A[14]. It also shows antitumor immunity effects in mice. Tumor growth was significantly suppressed in mice that were given propofol compared to with tumor growth in mice given saline. Therefore, it is concluded that propofol may have a beneficial effect on antitumor immunity in mice[15]. All of the above studies suggest that propofol possesses anti-cancer properties in addition to its sedative effects.

Dr. R. Siddiqui[16] and his colleagues from the Methodist Research Institute and Indiana University in Indianapolis, studied the effect of two omega-3 fatty acids , docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), combined with propofol on a breast cancer cell line *in vitro*. The results of the study showed that propofol and DHA or EPA has a much more significant effect on cancer cells when used in combination as conjugates, than when used alone. The conjugates inhibit cancer cell adhesion by 15% and 30% respectively, reduce cell migration by 50% and increase apoptosis by 40%. Harvey *et al*[17] reported the characterization and evaluation of novel anti-cancer conjugate, 2, 6-diisopropylphenol-docosahexaenoate (PP-DHA) and its analogs on breast cancer cell lines. Results of these studies suggest that these novel conjugates and their amide derivatives may be useful for the treatment of breast cancer.

Current trends in the treatment of human cancers favor drug combinations that result in improved responses, where the contributions of a variety of fatty acids have been proved highly significant[18, 19]. A number of fatty acids are part of our diet; therefore, nutritional dietary supplements highly enriched in certain fatty acid have been suggested to prevent the side effects of cancer therapy[19, 20]. Certain triglycerides and fatty acids have the potential to prevent or inhibit carcinogenesis[21-24].

We recently demonstrated the efficient synthesis of hydroxy fatty acid analog (particularly 9-hydroxy-11-Z-octadecenoic acid) of propofol (Scheme 1) and its significant *in vitro* selectivity against a panel of solid human tumor cell lines including HeLa, MCF-7 and HL-60(human leukemia) cells was also investigated. Its cytotoxicity was also determined against non-cancerous mammalian cells (VERO cells) for comparison. The compound was also screened for its antimicrobial activity against *E. coli*, *S. aureus* and *S. albus*.

Holarrhena antidysenterica, popularly known as ‘Indrajav’ is a shrub, distributed throughout India up to an altitude of 3,500 ft and even as far south as Travancore. It belongs to the family Apocynaceae. In Indian traditional medicine, this plant has been considered as a popular remedy for the treatment of dysentery, diarrhea, intestinal worms[25, 26]. Bark and seeds are used as a powerful antidysenteric, astringent, febrifuge, antihelmintic and antibacterial agents[26]. Seeds also possessed anti-diabetic activity[27, 28]. Bark and seeds contain alkaloids like conessine, kurchine, kurchcine, holarramine, conarrhimine, conamine, conessimine, isoconessimine, connesendine, conkurchine, kurchicine[29].

MATERIALS AND METHODS

Chemistry

All reactions were monitored by analytical TLC (Silica Gel “G”, E. Merck, India). Petroleum ether and diethyl ether (1: 1 v/v) was used as a developing solvent. Reaction products on TLC plates were visualized by UV light and by exposure to iodine vapors. The residue was purified by column chromatography, using silica gel G packing of particle size 60-120 mesh (petroleum ether/ diethyl ether, 1: 1 v/ v). ^{1}HNMR and ^{13}CNMR spectra were recorded on Advance DRX-200 Bruker, (Switzerland) NMR Spectrometer. Molecular weights were determined by MS route JMS-600H, Jeol (Japan) Mass Spectrometer. FTIR Spectra were recorded in chloroform on a Spectrum RX-1 FTIR, Perkin Elmer Spectrometer. All these analyses were done at CDRI (Central Drug Research Institute) Lucknow, India. 2, 6-diisopropyl phenol (propofol), dimethyl amino pyridine (DMAP) were procured from Acros chemicals. The coupling reagent- cyclohexyl carbodiimide (DCC) was purchased from Fluka chemical corporation (New York), and methylene chloride was purchased from CDH Chemicals (Mumbai, India). 9-Hydroxy-11-Z-octadecenoic acid (isoricinoleic acid) was isolated from Holarhena antidysentrica seed oil.

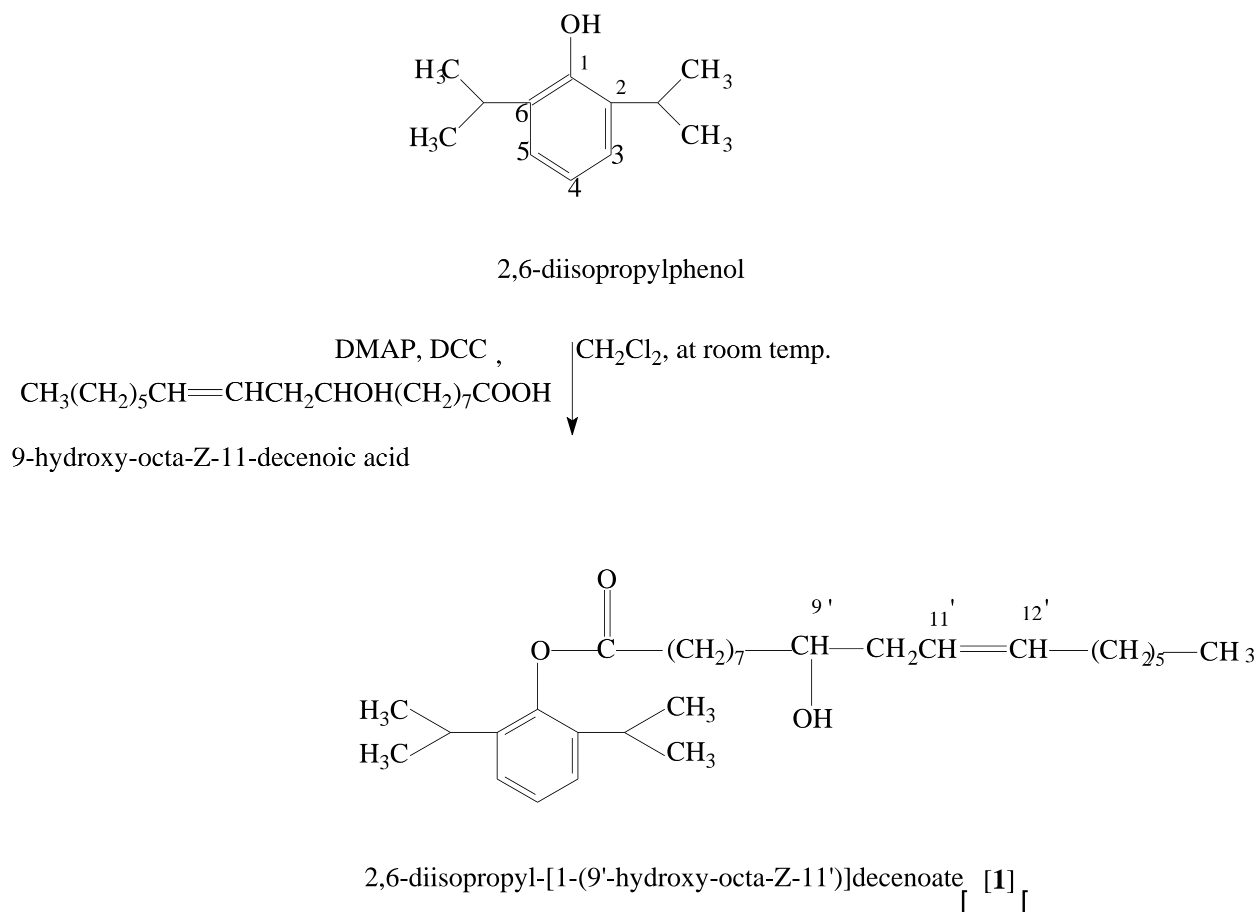
General procedure

Isolation of 9-hydroxy-11-z-octadecenoic acid.

The seeds of Indrajav were collected from local market of Aligarh city, India. Fresh seeds were dried at 37% in an incubator for 48 hours. Then the seeds were ground into powder in an electric grinder and were extracted with petroleum ether (30-60%). Solvent was removed from the oil on a rota vapor (rotary evaporator). By Gunstone Partition method[30], 9-hydroxy-11-z-octadecenoic acid was isolated from the above seed oil.

Synthesis

Equimolar amounts of propofol (1mmol) and 9- Hydroxy-11-Z-octadecenoic acid (1mmol) was dissolved in 5ml of methylene chloride, and DMAP (catalytic amount) was added to this. The reaction mixture was allowed to stir at room temperature under nitrogen for 10 minutes before DCC (1mmol) was added to it. The whole reaction mixture was allowed to stir at room temperature. The progress of reaction was monitored on TLC plates. This is a single product reaction and was completed in 12 hours. The reaction mixture was filtered to remove solid dicyclohexylurea and the filtrate was evaporated and the residue was passed through a silica gel column to obtain sticky, viscous and colorless oil (yield 95%). The chemical reaction involved is shown in SCHEME 1.



SCHEME 1

Assay for anti-cancer activity

In vitro screening of new drug candidate against human cancer cell line panel is carried out and results are tabulated in Table 1. All the cell lines are from the American type Culture Collection (ATCC), USA. The cells were cultured in 75 cm^2 culture flask, supplemented with bovine calf serum and amikacin at 37°C, human culture

techniques. Cell counts were made after 48hrs of incubation. Cells were seeded to the wells of the plate at a density of 25,000 cells/well and were allowed to grow for 48hrs at 37°C. Diluted samples were added to cells and again incubated for 48hrs at 37°C. The number of viable cells was determined using modified Neutral Red assay procedure. Cells were washed with saline and incubated for 90 minutes with the medium containing Neutral Red. IC_{50} (the concentration of the test compound that caused a growth inhibition of 50% after 48hrs of exposure of the cells) were calculated from the dose curves generated by plotting % growth v/s the test concentration on a logarithmic scale. All the assays were performed in triplicate and then mean values will be considered.

Assay for antimicrobial activity

The *in vitro* antimicrobial activity was carried out against *E. coli, S. aureus* and *S. albus.* These strains were streaked on nutrient agar plates separately and grown overnight. Single-well isolated colonies of each type of bacteria were incubated in separate nutrient mediums for 16 h at 37 °C for the experiment. To determine the zone of inhibition cup-plate method was employed[31]. In this technique bacteria liquid culture of each type grown in log phase was added aseptically to the autoclaved LB agar medium maintained at 45 °C, mixed well and poured immediately into sterile Petri dishes separately. After solidification, wells of about 6 mm were cut into agar plates aseptically. Solution of 100 µg/ml of compound was prepared in DMF. Standard antibiotic Chloromycetin was screened under similar conditions. 100 µl of this solution was added to each well and incubated at 37 °C. One of the wells was used as control by adding 100 µg/ml of DMF. Zone of inhibition was measured in mm after 24 h and compared with the standard drug.

RESULTS AND DISCUSSION

***Characterization of 9-hydroxy-11-Z-octadecenoic acid* (from seed oil of *Holarrhena antidysenterica*)**

Viscous oil, $R_F = 0.2$, isolated yield, 95%.
IR ($CHCl_3$, cm^{-1}): 3418.0, 3013.0, 2930.4, 2858.4, 1710.8, 1640.0, 1460.5, 1216.9, 1104.4, 932.6, 763.5, 668.8.
MS-EI found $[M+H]^+$ 298.4638; $C_{18}H_{34}O_3$ $[M+H]^+$ requires 298.4659.
^{1}HNMR ($CDCl_3$, ∂_H, ppm): 0.89(t, J= 6Hz, 3H of terminal –CH_3), 3.39(s, 1H, C<u>H</u>-OH), 3.62(s, 1H, O<u>H</u>), 5.37(m, 1H, -CH=C<u>H</u>), 5.84(m, 1H, -C<u>H</u>=CH), 3.81-4.47(m, 4H), 2.13(m, 4H), 2.33-2.63(m, 6H), 1.31-1.83(m, 12H).
^{13}CNMR ($CDCl_3$, ∂_c):14.03, 22.53, 23.5, 24.6, 25.46, 27.14, 29.12, 31.83, 33.9, 35.86, 37.24, 42.8, 129.07, 130.63, 179.46.

Spectral studies of the compound [1]

Viscous oil, $R_F = 0.5$, (petroleum ether/diethyl ether, 1:1 v/v as a developer), isolated yield, 90%.
IR ($CHCl_3$, cm^{-1}): 3429.2, 3012.6, 2931.3, 2859.3, 1744.0, 1694.8, 1646.6, 1525.4, 1383.7, 1372, 1216, 1164, 1098.5, 930.7 and 754.7.
MS-EI found $[M+H]^+$ 458.7287; $C_{30}H_{50}O_3$ $[M+H]^+$ requires 458.7297.
^{1}HNMR ($CDCl_3$, ∂_H, ppm): 0.88(t, J=6.4Hz, 3H), 1.182(d, J=6.6Hz, 6H), 1.27, 1.248(d, J=6.6Hz, 6H), 2.615(m,6H), 2.40(m,3H), 2.92(m,2H), 3.20(m,2H), 3.615(m,1H), 3.90(m,2H), 4.22(m,2H), 5.38(m,1H), 5.673(m,1H) 6.894(m,1H), 7.042(d, J=7.5Hz, 1H), 7.230(d, J=8.1Hz, 1H), 1.308-2.14 (m, 12H).
^{13}CNMR ($CDCl_3$, ∂_c): 14.47, 22.72, 23.53, 24.95, 25.34, 26.29, 26.98, 27.44, 29.33, 31.46, 32.67, 34.10, 35.77, 37.35, 38.64, 49.65, 55.96, 68.08, 71.61, 73.60, 120.45, 123.30, 123.78, 126.34, 132.5, 149.97, 154.06, 172.34.

After isolation of 9-hydroxy-11-Z-octadecenoic acid from the seed oil of *Holarrhena antidysenterica*, it was characterized by various spectroscopic techniques including IR, ^{1}HNMR, ^{13}CNMR and Mass spectroscopy. The IR spectra of the compound revealed strong absorption bands at 1710.8 cm^{-1} and 1216.9 cm^{-1} corresponding to C=O and C-O bonds respectively, indicating the presence of carbonyl carbon. C^{13}NMR studies also confirmed

the presence of carbonyl carbon showing carbon signal at ∂_C 179.46. Presence of hydroxyl group was confirmed by absorption band at 3418.0cm^{-1}and its respective carbon signal appeared at ∂_C 71.77 which is further correlated with proton signal showing chemical shift at ∂_H 3.625ppm (s, 1H). IR spectra revealed a sharp band at 1640.0 cm^{-1} indicating the presence of double bond which is further related to chemical shifts at ∂_H 5.37(m, 1H) and 5.84(m, 1H) ppm for the two olefin protons 11H and 12H respectively and their respective carbon signals appeared at ∂_C 129.07 and 130.63. The bands at 2930.4 and 2858.4cm^{-1} correspond to the aliphatic CH bonds. Some significant signals appeared at ∂_H 0.89(t, J= 6Hz, 3H of terminal CH_3 group), 3.39(s, 1H, CH-OH) and 1.31-1.83(m, 12H) for the rest of the fatty acid chain length. Few other carbon signals appeared at ∂_C 14.03(C-18), 22.53(C-17). This compound has a methylene interrupted 9-hydroxy and Z-11- olefin system in its C-18 fatty acid moiety. The IR spectrum of the conjugate revealed broad, strong absorption bands at 1744.0 cm^{-1} and 1216cm^{-1}which are attributable to C=O and C-O bonds, respectively, and indicate the presence of an ester, which was also confirmed by the presence of a significant carbon signal at ∂_C 172.32 showing the presence of carbonyl group. A strong band at 3429.2 cm^{-1}indicate the presence of hydroxyl group which was further confirmed by the chemical shift of 9'-H at ∂_H 3.615ppm, and its respective carbon signal appeared at ∂_C 71.61. The band at 3012.6cm^{-1} is characteristic of an aromatic C-H (propofol) and the band at 2931.3 and 2859.3 cm^{-1} is characteristic of aliphatic C-H bonds. A distinct band at 1646.6 cm^{-1} shows the presence of alkene. The two olefin protons, 11'H and 12'H were observed at ∂_H 5.38ppm and 5.673ppm and correlated with observations at ∂_C 126.34 and 132.5 respectively. A few other significant carbon signals for the acyl chain were recorded at ∂_C 14.47 (C-18'), 22.72 (C-17'), 25.34 (C-3'), 34.1 (C-2'). The chemical shifts for aromatic protons are moved downfield at ∂_H 6.894 (m, 1H), 7.067(d, J= 7.5Hz, 1H), 7.230 (d, J=8.1Hz, 1H) and their respective carbon signals appeared at ∂_C 120.45, 123.30, 123.78. For 12 protons of the two isopropyl groups, two doublets were observed at ∂_H 1.182 (d, J=6.6Hz, 6H) and 1.248(d, J=6.6Hz, 6H) and their respective carbon signals appeared at ∂_C 23.53 and 24.95. Two multiplets for protons of carbon atoms adjacent to carbon number 2 and 6 were observed at ∂_H 2.921ppm and 3.20ppm and their respective carbon signals were appeared at ∂_C 26.29 and 26.98.The compound 1-isopropyl-9'-hydroxy-octa-Z-11'-decenoate was examined for its *in vitro* cytotoxicity against a panel of solid human tumor cell lines including HeLa, MCF-7 and HL-60(human leukemia) cells. Its cytotoxicity was also determined against non-cancerous mammalian cells (VERO cells) for comparison. A number of studies have concluded that chemically modified FA molecules possess more specific and potent biological activity with possible changes in their therapeutic targets[32]. The present work is based on the chemically transformed FA analog of propofol. The compound was cytotoxic against all cancer cell lines where as no effect was observed against normal cells (VERO cells) up to the highest concentration of 15µM in the assay, thus demonstrating selectivity towards the tumor cells. The cytotoxic potency of this compound is expressed in terms of IC_{50} values as shown in Table 1. The significantly higher anti-cancer activity of this compound is attributed to the presence of a methylene interrupted 9-hydroxy and Z-11- monounsaturation in its C-18 fatty acid moiety.

Table1 Anti-cancer activity of compound [1]

Compound[a]	Cell lines[b]	IC_{50}, µM
1	HeLa	0.42
	MCF-7	2.10
	HL-60	0.31
	VERO	NA

[a]*The highest concentration tested was 15 µM.*
[b]*NA, not active*; *HeLa, human cervical epitheloid carcinoma; MCF-7, human breast adenocarcinoma; HL-60, human leukemia; VERO, monkey kidney fibroblasts*.

The compound was also screened for its antimicrobial activity against *E. coli*, *S. aureus* and *S. albus*. The compound show good antimicrobial activity against *E. coli* and *S. albus*. Results of anti-microbial screening are reported in Table 2.

Table 2. Anti-microbial activity of compound [1]

	Diameter of zone of inhibition		
Compound	E.coli	S. aureus	S. albus
1	15mm	NA	10mm
Chloromycetin	22mm	18mm	20mm

DMF used as the control; concentration = 100µg/ml of DMF; NA, not active.

CONCLUSION

These results suggest that the novel 1-isopropyl-9'-hydroxy-octa-Z-11'-decenoate conjugate reported here may be useful for the treatment of cancer as it show promising anti-tumor activity against a panel of solid human tumor cell lines. The conjugate also showed significant anti-microbial activity against E. coli and S. albus. Further investigations on this novel compound may provide a useful lead in the development of new pharmaceutical products.

ACKNOWLEDGEMENTS

We are thankful to the Chairman, Department of Applied Chemistry, Faculty of Engineering and Technology, for providing all the facilities and support. We would also like to thank Microbiology section of JNMC, A.M.U. for helpful biological activity and CDRI, Lucknow for providing spectral data.

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